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PATHOGENIC AND NONPATHOGENIC STRAINS OF ENTAMOEBA HISTOLYTICA CAN BE DIFFERENTIATED BY MONOCLONAL ANTIBODIES TO THE GALACTOSE-SPECIFIC ADHERENCE LECTIN

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Pathogenic determinants found on the outer surfaces of microorganisms determine their ability to adhere to and/or invade mammalian tissues. The galactose lectin produced by Entamoeba histolytica provide the basis for development of a model system for the environmental detection of adherence and invasive properties harbored by potential threat agents. Monoclonal antibodies (mAb) directed against the 170 kDa heavy subunit or the amebic lectin defined six nonoverlapping epitopes. We tested 50 pathogenic and nonpathogenic strains of Entamoeba histolytica for reactivity to these mAb. By radio immunoassay mAb to epitopes 1 through 6 reacted in the radioimmunoassay with all 16 pathogenic strains tested. However only mAb to epitopes 1 and 2 bound to the lectin from nonpathogenic Entamoeba histolytica. A lectin radioimmunoassay based on mAb to epitopes 1 and 3 proved to be a rapid and simple means to distinguish pathogenic from nonpathogenic amebae. In addition, mAb to epitopes 2 and 4 were covalently coupled to microparticles and specifically distinguished solubilized antigens of nonpathogenic pathogenic strains.

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PREFACE

The work described in this report was authorized under Project No. 1FJ1X2XXRPEW and Contract No. DAAD0590P5162. This work was started in March 1990 and completed in March 1991. The work was performed at the U.S. Army Chemical Research, Development and Engineering Center (CRDEC) and the University of Virginia. The experimental data are on record at both locations.

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This report has been approved for release to the public.

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PATHOGENIC AND NONPATHOGENIC STRAINS OF *ENTAMOEBA HISTOLYTICA* CAN BE DIFFERENTIATED BY MONOCLONAL ANTIBODIES TO THE GALACTOSE-SPECIFIC ADHERENCE LECTIN

Introduction

Entamoeba histolytica is the protozoan responsible for amebic colitis and liver abscess in humans. *E. histolytica* is spread by the fecal-oral route, infection occurring via ingestion of the organism. *Entamoeba histolytica* infects an estimated 480 million individuals annually, of which 10% develop colitis or liver abscess (18). *E. histolytica* strains cultured from patients with invasive disease have been classified into pathogenic zymodemes or strains on the basis of their distinctive hexokinase and phosphoglucomutase isoenzymes. Pathogenic zymodeme amebae are indistinguishable morphologically from the nonpathogenic zymodeme *E. histolytica* (14). Isoenzyme analysis has been performed on a research basis on over 3000 isolates. A nonpathogenic zymodeme has never been cultured from a patient with invasive amebiasis, and only rarely have pathogenic zymodemes been isolated from asymptomatically colonized individuals (12-15). The reasons for the apparently great difference in virulence of these *E. histolytica* zymodemes, and the search for practical clinical tests to distinguish them, are areas of intense investigation.

The galactose binding lectin has been purified from a pathogenic zymodeme (axenic strain HM1:IMSS) of *E. histolytica*. It is a 260 kDa heterodimeric glycoprotein consisting of a 170 kDa heavy subunit linked by disulfide bonds to a 35 kDa light subunit (7-9). This lectin mediates in vitro adherence to human colonic mucin glycoproteins, suggesting that it is involved in the initial colonization and invasion of the colon (3). Six antigenically and functionally distinct epitopes have been mapped on the heavy subunit with monoclonal antibodies (mAb) (a). Here we use these mAb to demonstrate that the lectin is present in nonpathogenic zymodemes, but that it contains only two of the six epitopes identified on the pathogenic lectin.

Materials and Methods

Cultivation and harvesting of E. histolytica. Axenic *E. histolytica*, pathogenic strain HM1-IMSS, were grown in medium TYI-S-33 (trypticase yeast extract, iron, and serum) with 100 U/ml of penicillin and 100 µg/ml of streptomycin sulfate (Pfizer, Inc., New York, NY) at 37°C in 250-ml plastic tissue culture flasks (8,10). Amebae were harvested after completion of log phase growth at 72 h of by centrifugation at 150 g for 5 min at 4°C and washed twice in ice cold 75 mM Tris (Sigma Chemical Co., St. Louis, MO) 65 mM NaCl, pH 7.2 (8,10). Non-axenized pathogenic and nonpathogenic strains were grown in TYSGM-9 medium containing rice starch in the presence of bacterial flora. In a few cases nonpathogenic amebae were grown in Robinson's media or TYI-S33 containing bacterial associates (2,11). The bacterial associates were not identified in any of the cultures. The amebic strains were classified into pathogenic and nonpathogenic zymodemes by thin layer starch gel electrophoresis followed by visualization of the hexokinase and phosphoglucomutase isoenzyme bands (12 & 13). Amebae were washed in 75 mM Tris 65 mM NaCl pH 7.5, pelleted and lyophilized in order to transport them to the United States for RIA. Pathogenic amebae utilized included HM1:IMSS, SAW 755, Rhaman, Thailand 078 and 090, NIH 303 and 200 and nine new isolates from South Africa. Nonpathogenic amebae tested included WI-1285:I, CDC:0784:4, SAW 760 and 1734, and over 20 new isolates from South Africa, plus the Laredo strain.

Monoclonal antibodies. The production and epitope mapping of murine anti-lectin mAb have been previously reported elsewhere (8,9). The epitope specificities and designations of the mAb used were: epitope 1, 3F4; epitope 2, 8A3; epitope 3, 7F4; epitope 4, 8C12; epitope 5, 1G7; epitope 6, H85. The mAb were purified from 50% ammonium sulfate fractions of ascites by protein A affinity chromatography or preparative isoelectric focusing (4). Purified mAb were labeled with ^{125}I using the chloramine T technique to a specific activity of 20 to 40 $\mu Ci/\mu g$ (4).

Radioimmunoassay of the lectin. Polyvinylchloride microtiter plates (Dynatech, Alexandria, VA) were coated with 1 μg/well of anti-lectin monoclonal antibody 3F4 (epitope 1) in 0.1 M bicarbonate buffer, pH 9.6, overnight at 4°C and residual binding sites blocked with 1% bovine serum albumin in phosphate-buffered saline, pH 7.2, containing 0.05% Tween 20. Affinity-purified lectin (1-50 ng/well) or amebae solubilized in 50 mM Tris, 250 mM NaCl, 0.5% NP-40 pH 8.0 were incubated in the antibody-coated wells for 2 hr at room temperature. After washing the wells immobilized lectin was quantified by adding 10^5 cpm/well of ^{125}I-labeled anti-lectin monoclonal antibody 7F4 (epitope 3) for 4 h at room temperature (8). Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Recognition of pathogenic and nonpathogenic amebae by RIA. The ability of ^{125}I-labeled mAb directed against epitopes 1-6 to bind to the lectin from the different *E. histolytica* strains was tested by RIA. Polyvinyl chloride microtiter plates were coated with mAb 3F4 (epitope 1) and blocked as described above. Serial dilutions of solublized amebae were added to the wells and incubated for 2-4 h at room temperature. The plates were then washed and 1-2 x 10^5 cpm of ^{125}I mAb specific for epitopes 2-6 added for an additional 2-4 h incubation. After further washing the plates were dried and individual wells counted in a gamma counter. Standard curves for the lectin were performed with each of the ^{125}I-labeled mAb, and final results expressed as pg lectin detected/10^3 amebae.

Materials for latex agglutination: One micron diameter carboxylated microparticles (2.5% latex solids) containing a fluorescent dye (Polysciences, Inc., Warrington, PA.) were used as a support to which purified monoclonal antibodies were covalently coupled. Monoclonal antibodies to epitope 1, 3F4; epitope 2, 8A3; and epitope 4, 8C12 were used. Carbodiimide coupling reagents and procedures from Polysciences were followed, varying the microparticle/protein ratio. Quantigold protein determinations (Diversified Biotech, Newton Centre, MA) were performed on supernatents of each experimental coupling, along with a standard curve based on increasing nanogram concentrations of bovine serum albumin (BSA), to determine percent retention of protein to microparticles. The amebic strains used in the agglutination assays included the nonpathogenic strain, SAW 760, and the pathogenic strain, HM-1. Lyophilized preparations were solubilized in 50 mM Tris buffer pH 8.3 containing 150 mM NaCl, 0.5% tritonX 100, 5 mM EDTA, and 2 mM PMSF.

Protein coupling to microparticles: Microparticles (2.5% latex solids) were placed in a microcentrifuge tube and washed by centrifugation for 6 minutes in 0.1 M carbonate buffer, pH 9.6. This procedure was repeated once, the pellet then resuspended in 0.02 M sodium phosphate buffer, pH 4.5. This wash was repeated twice, using phosphate buffer, and the pellet redispersed in 0.6 ml of sodium phosphate buffer. A fresh 2% carbodiimide solution in sodium phosphate buffer was prepared and 0.6 ml added dropwise to the microspheres. This combination was mixed for 3.5 to 4.0 hours on a rotary shaker, centrifuged for 6 minutes and the supernatent discarded. The pellet was resuspended in 0.2 M borate buffer, pH 8.5, centrifuged and washed twice more. The pellet was resuspended in 1.2 ml borate buffer and 200 micrograms of affinity purified antibody was added. The preparations were mixed gently

on a rotary shaker overnight at room temperature. After centrifugation for 10 minutes, the supernatent was carefully removed and retained for protein determination. The pellet was resuspended in one ml of 0.1 M ethanolamine in borate buffer and gently mixed at room temperature for 30 minutes. After centrifugation and discarding of the supernatent, the pellet was resuspended in 1 ml of 10 mg/ml purity grade BSA (FisherBiotech, Fair Lawn, N.J.) in borate buffer. This was mixed gently for 30 minutes at room temperature, centrifuged, and the process was repeated once. Following a 6 minute centrifugation, the pellet was resuspended in 1 ml of storage buffer (0.1 M phosphate, pH 7.4, 10 mg/ml BSA, 5% glycerol, and 0.1% sodium azide) and stored at 4°C.

Results

RIA using epitope 1 and 3 mAb is specific for pathogenic E. histolytica. We previously reported the development of a two site mAb based assay for the 170 kDa subunit of the galactose lectin. In this assay the lectin is "captured" by epitope 1 mAb 3F4 coated on microtiter wells and detected by ^{125}I labeled epitope 3 mAb 7F4 (8). The amount of radioactive 7F4 bound per well is linearly related to the amount of lectin added to the well from 1 to 25 ng/well. The RIA is capable of detecting as few as 30 pathogenic strain HM1:IMSS amebae or 0.5 ng of antigen added to a well (Figure 1 A&B). In contrast, no lectin could be detected by this RIA in the nonpathogenic zymodeme CDC:0784:4, even with 1000 amebae/well (Figure 1B). The failure of this RIA to detect lectin in the CDC:0784:4 was not due to the different culture conditions required for the nonpathogenic zymodeme. No significant difference in lectin content was detected when pathogenic HM1:IMSS amebae were grown in TYI-S-33 medium without or TYSGM-9 medium with bacterial associates from the CDC:0784:4 culture (7.7 vs 4.8 pg/ameba respectively).

A total of 18 pathogenic and 32 nonpathogenic zymodeme strains were assayed with the epitope 1 and 3-based RIA. Lectin was detected in all of the pathogenic strains and none of the nonpathogenic strains (Figure 2). The absolute quantity of lectin detected per ameba varied greatly amongst the pathogenic amebae, however the mean lectin concentration of the pathogenic amebae remained significantly different from the undetectable lectin concentration in the nonpathogenic amebae ($p < .01$). Amebae with lower lectin concentration generally were the strains that had been frozen, lyophilized and sent from South Africa to the United States prior to RIA, so that the lower lectin concentrations detected could be due to instability in the lectin during transport. No correlation could be made between lectin concentration and culture medium (TYI-S-33, TYSGM-9, Robinson's) used.

RIA using epitope 1 and 2 mAb detects pathogenic and nonpathogenic E. histolytica. The inability of the RIA using epitope 1 and 3 to detect lectin could reflect either an absolute lack of lectin or a loss of mAb binding to the nonpathogenic lectin. The two site mAb-based RIA was therefore modified so that ^{125}I labeled epitope 2 mAb 8A3 was used to detect lectin captured in the epitope 1 mAb 3F4 coated wells. This RIA had similar sensitivity to the epitope 1 and 3 assay, with the amount of radioactive 8A3 bound per well linearly related to pathogenic lectin concentrations under 50 ng/well (data not shown). The assay in this configuration detected lectin in the nonpathogenic as well as the pathogenic zymodemes, indicating that the lectin was present but no longer recognized by epitope 3 mAb. The amount of lectin per ameba showed generally the same variations as seen with the pathogenic amebae (Figure 3).

Antigenic conservation of epitopes 1-6. The binding of radiolabeled mAb to all six of the epitopes on the 170 kDa subunit was measured by RIA. All six epitopes defined by the mAb were present in 16/16 pathogenic zymodemes tested. These pathogenic strains included HM1:IMSS, SAW 755, Rhaman, Thailand 078, Thailand 090 and nine new isolates from

South Africa (seven from liver abscess patients and two from asymptomatically colonized individuals). Epitopes 1 and 2 were present and 3-6 absent in all of the 27 nonpathogenic isolates tested, including strain SAW 1734 and 26 recent isolates (assayed within several weeks of initial culture) from colonized individuals in South Africa. Nonpathogenic strains CDC 0784:4 and SAW 760 reacted with mAb against epitopes 1 and 2 but not 3; epitopes 4-6 were not tested on these 2 strains.

Microparticles with monoclonal antibody to epitope 1, 8A3; and epitope 2, 3F4 covalently bound were mixed with solubilized antigen from amebic strains SAW-760 and HM-1 and observed for agglutination. Twenty microliter amounts of the preparations, each with solubilized amoeba, undiluted (equivalent 10^6 per ml), and at 1:2, 1:10, 1:100, 1:1000, and 1:10000, were observed against a black background. Protein determinations on the supernatents revealed 68.1% retention for mAb 3F4 and 61.6% retention of 8A3. Although both monoclonals were handled the same and coupled in the same experimental run, good results were obtained with 8A3 but not with 3F4 as shown in Table I. Controls, microparticles with no antibody, did not cluster when mixed with solubilization buffer. Each amebic preparation with buffer alone showed no clumping. A confirmatory follow-up using 8A3, and in addition, antibody to epitope 4, BC12 (pathogenic only) gave specific, clear reactions as shown in Table 2. Optimal ratios for the covalent coupling procedure were, 0.4 ml (2.5% solids) of microparticles, 200 micrograms antibody in 1.524 ml reaction mixture, obtaining 61.6% protein retention.

Discussion

Monoclonal antibodies against the amebic lectin have proven to be an excellent means to distinguish pathogenic from nonpathogenic zymodemes of *E. histolytica*. The data presented here show a perfect correlation of mAb reactivity with zymodemes for all 50 strains tested, including isolates from 3 continents. The remarkable antigenic conservation of the epitopes defined by the murine mAb on the lectin from pathogenic zymodemes offers the promise of worldwide applicability of this antigen-detection test. While zymodeme classification has been the standard epidemiologic tool for over a decade, it is a lengthy and complex procedure that has not been useful in clinical practice. Generally several days of laboratory work are required to determine the zymodeme after already an average of two days spent to establish a culture from the specimen. The rapidity of the anti-lectin RIA was demonstrated by its accurate assignment of zymodemes to 35 fresh clinical isolates from South Africa. We have adapted the RIA to a more practical ELISA format and plan to test the feasibility of direct testing of stool specimens, with the hope of eliminating even the two days required to establish cultures.

Strachan et al have reported a mAb to an undefined antigen which by immunofluorescence distinguished pathogenic from nonpathogenic amebae (15). The RIA that we have developed is quantitative, to a well defined antigen, and is easier to perform and automate than immunofluorescence. DNA probes which differentially hybridize to pathogenic and nonpathogenic zymodemes have been developed by Garfinkel et al (6) and Tannich et al (16). Only with further testing and development will it be determined whether antigen detection or DNA probes are better suited for the diagnosis of amebiasis. What is clear is that these recent developments in diagnosis offer significant advantages over the presently cumbersome microscopic identification of *E. histolytica* performed in clinical microbiology laboratories today. With the additional ability of these new tests to determine whether the infecting *E. histolytica* is pathogenic or nonpathogenic, potentially toxic and expensive anti-amebic chemotherapy could be reserved for the minority of individuals who are infected with pathogenic zymodemes.

TABLE 1. Agglutination of antibody coated microspheres with solubilized amebic antigen.

Antibody	Dilution	Amebic strain HM-1	Amebic strain SAW-760	Final conc equiv amoeba in rct mix
8A3	Undil	4+	4+	2×10^4
	1:2	2+	4+	1×10^4
	1:10	1+	2+	2×10^3
	1:100	ft	1+	200
	1:1000	ft	ft	20
3F4	No agglutination with either strain at any dilution			

4+ - Readily occurring strong agglutination
3+ - Readily occurring moderately strong agglutination
2+ - Distinct agglutination
1+ - Delayed weak agglutination
ft - Faint agglutination, difficult to confirm

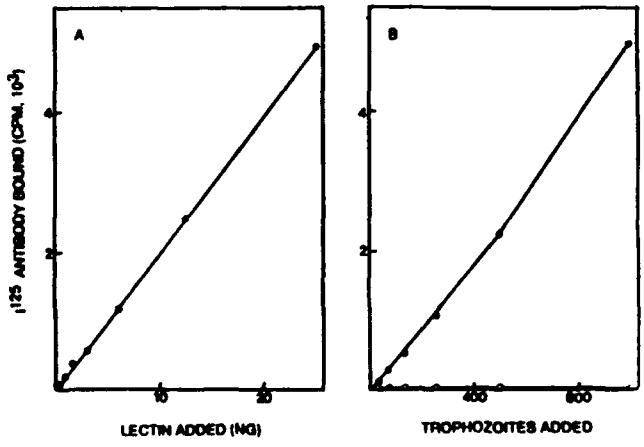

FIG. 1. RIA of the galactose–*N*-acetylgalactosamine adherence lectin. Microdilution wells were coated with anti-lectin MAb 3F4 (epitope 1). Known amounts of the purified lectin or detergent-solubilized *E. histolytica* trophozoites (●, pathogenic zymodeme strain HM1:IMSS; O, nonpathogenic zymodeme CDC:0784:4) were added to the 3F4-coated wells. The amount of lectin bound to the 3F4-coated wells was quantitated with ^{125}I-labeled anti-lectin MAb 7F4 (epitope 3).

TABLE 2. Specific confirmatory agglutination of equivalent 2 x 10^4 amebic preparations with antibody coated microspheres.

Antibody/ microsphere	Microliters used	Buffer/ PMSF	Amebic strain HM-1	Amebic strain SAW-760	Result
8A3	20ul	/	20ul	/	3+
Control	20	20ul			Neg
8A3	20	/	/	20	2+
Control	20	20	/	/	Neg
8C12	20	/	20	/	3+
Control	20	20	/	/	Neg
8C12	20	/	/	20	Neg
Control	20	20	/	/	Neg

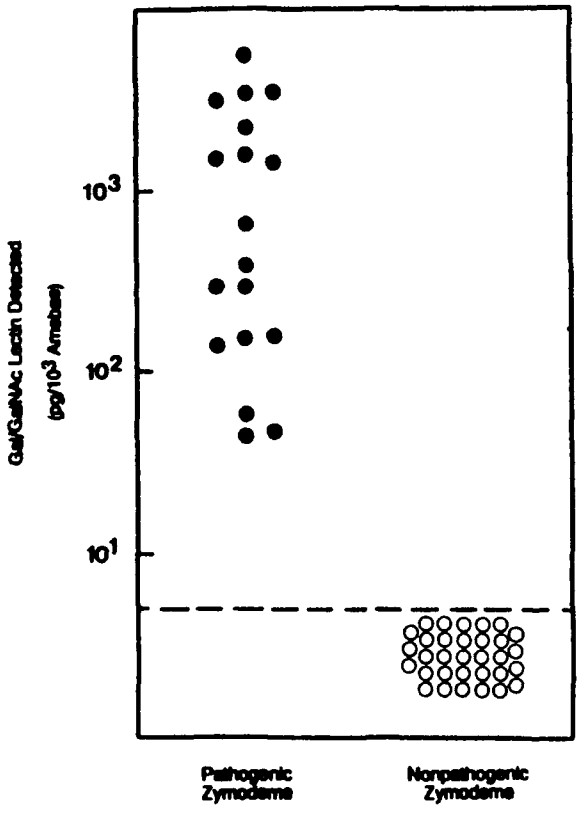

FIG. 2. RIA with MAb to epitopes 1 and 3 of the galactose-*N*-acetylgalactosamine (Gal/GalNAc) lectin performed on pathogenic and nonpathogenic *E. histolytica*. Solubilized amebic proteins were added to wells coated with anti-lectin MAb 3F4 (epitope 1). The amount of lectin bound to the wells was quantitated with ^{125}I-labeled anti-lectin MAb 7F4 (epitope 3). Symbols: ●, pathogenic zymodeme; ○, nonpathogenic zymodeme. The broken line represents the limit of sensitivity for the RIA.

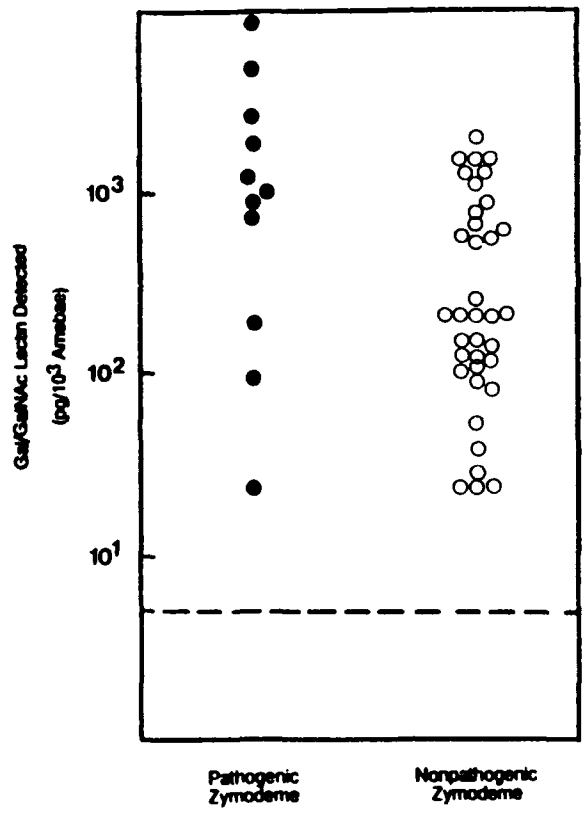

FIG. 3. RIA with MAb to epitopes 1 and 2 of the galactose-*N*-acetylgalactosamine (Gal/GalNAc) lectin performed on pathogenic and nonpathogenic *E. histolytica*. Solubilized amebic proteins were added to wells coated with anti-lectin MAb 3F4 (epitope 1). The amount of lectin bound to the wells was quantitated with ^{125}I-labeled anti-lectin MAb 8A3 (epitope 2). Symbols: ●, pathogenic zymodeme; ○, nonpathogenic zymodeme. The broken line represents the limits of sensitivity for the RIA.

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Initial steps in providing an avenue for separating pathogenic from nonpathogenic strains using latex agglutination look promising. We have shown specific, reproducible agglutination using 8A3, a mAb directed against epitope 2 which is found on both pathogenic and nonpathogenic strains. In addition, microparticles coated with mAb 8C12, directed against epitope 4, reacted specifically with pathogenic strains and did not cross react with nonpathogenic strains. Therefore, with successful conversion of this technique to use with processed liquid specimens, a discriminatory method for use in the clinical lab, at the bedside, or in the field is plausible.

It is not immediately evident why good results were obtained using mAb to epitope 2 but not mAb to epitope 1. Repeated attempts to couple 3F4 to microspheres (data not shown) failed, whereas coupling of 8A3 gave consistently good results. It is known that net charge of the molecule is important during all steps of the coupling process and pH can effect this charge. Each antibody is unique in its conformation and reactivity and must be treated as an individual molecule in relation to expectations for successful coupling. Hidden agendas to consider during immobilization include antibody disorientation; deformability of antibody molecules as the immobilization procedure progresses; concentration overload; number, density, and spacing of COOH groups on the surface of the microparticles; and crosslinking of the antibodies by agents such as ethanolamine, which is not adequately removed in the wash steps. It is interesting to note that the best agglutination was obtained when 60 - 70% of the protein used was retained as opposed to 90% (data not shown). This may reflect a surface concentration of antibody too high to effectively couple the amebic antigen.

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